# THE PRESERVATION OF YELLOW FEVER VIRUS*

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(Received for publication, March 28, 1929)

In preparation for a comparative study of the characteristics of strains of yellow fever virus from West Africa and Brazil, we found it necessary to seek a reliable method of preserving the virus over a considerable period of time. The ideal method for our purposes would be one which would permit the sending of active virus in small sealed containers on sea voyages lasting over a month, and would also allow storage in the laboratory for several months without serious loss of virulence. Such a method was found among several processes previously applied to the preservation of other viruses, and it has now been in use long enough in connection with our experiments to show that it can be depended upon.

In the first work with experimental yellow fever in monkeys, Stokes, Bauer and Hudson (1) kept the virus alive for a time by passing it directly from animal to animal. They made inoculations by taking blood from the sick monkey, adding it to a citrate solution to prevent clotting, and injecting the mixture into a healthy animal. This method would have required an excessive number of monkeys to preserve even one strain of the virus during an extended study. The interval between successive inoculations had to be short for several reasons: the citrated blood could not be stored more than a few days without danger of losing the virus, the incubation period was usually brief, and the blood for transmission was drawn as a rule on the first day of illness. The difficulty was later diminished by these investigators by transferring the infection from monkey to monkey by means of mosquitoes, and thus lengthening to several weeks the time interval between successive monkeys in a series. This method is far from ideal, however, for the mosquitoes require much care and occasionally develop a high

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation. Through the courtesy of The Rockefeller Institute in New York laboratory facilities were made available.

mortality which might result in the loss of the yellow fever strain. The use of mosquitoes, moreover, necessitates special equipment and adds an extra hazard for the experimenters.

Sellards (2) succeeded in preserving yellow fever virus in frozen monkey liver for twelve days while transporting it from West Africa to England. Keeping tissues in a frozen state requires conditions difficult to arrange, especially on long voyages. The method, however, was one which promised to be highly useful, if tests proved it reliable for extensive periods, especially where the use of mosquitoes is impracticable.

### *The Yellow Fever Strains Used*

For the purpose of comparing the values of different methods of preserving yellow fever virus we have brought together in tables the results of our observations with two strains which proved to be practically identical in their characteristics and very satisfactory for experimental work. Both were highly virulent and fairly constant in their effects. For one, the "French" strain ("F" in the tables), we are indebted to Dr. A. W. Sellards, who had brought it to America from West Africa via England. It had been transmitted to monkeys by Mathis, Sellards, and Laigret (3). The other is the "Asibi" strain ("A" in the tables), with which Stokes, Bauer, and Hudson did most of their work. It was originally obtained on the Gold Coast, West Africa, and was sent to us from Lagos, Nigeria, by Dr. Henry Beeuwkes, Director of the West African Yellow Fever Commission of the Rockefeller Foundation.

### *Sources of the Virus*

In each instance the virus studied was preserved in the blood or the liver tissue of *Macacus rhesus* monkeys.

After an animal was inoculated, the rectal temperature was taken twice a day, and when the temperature rose to 40°C. for the first time, a specimen of blood was taken. The animal was anesthetized with ether and the blood was drawn from the heart.

The sick animals were allowed to die or were chloroformed when moribund. When needed as a source of virus, liver tissue was removed at necropsy with precautions for sterility. In all cases where an animal is reported as having died of yellow fever, the gross lesions of this disease were found on necropsy, and characteristic changes were seen in microscopic preparations of tissues, especially those of the liver. In a few instances the presence of tuberculosis or dysentery com-

plicated the picture and made it difficult to determine the onset and course of the yellow fever. Such cases were excluded from consideration in this study.

Subcutaneous or intraperitoneal injections of fresh first-day blood were almost invariably followed by the development of experimental yellow fever, even when small doses were used. As results with material which had been kept less than nine days are of little interest to this study, they have been omitted from the tables presented, except for the two partial titrations of fresh citrated blood of Monkeys 95 and 118 included in Table I. All injections of virus into monkeys in this study were intraperitoneal except in the case of the two titrations, in which they were subcutaneous. We have noticed no difference between the results following these two methods of inoculation.

The liver tissue proved less reliable for inoculation than the first-day blood. This is as would be expected when one considers that the virus diminishes rapidly in the blood after the first day or two of fever, and probably decreases in the liver during the latter days of the disease. The duration of the illness varies in the different cases and the concentration of virus in the liver at death probably varies also. Whatever the explanation, the liver tissue, though usually infectious, proved to be less consistently so than the first-day blood specimens.

Except when specially noted in the tables, no specimens which failed to infect are included unless the original fresh material was proven infectious by the inoculation of some other specimen derived from it. In a very few cases the inoculated monkeys had previously received material related to yellow fever, most often human blood two or three weeks old, and had shown no reaction. On account of the remote possibility that the animals had been protected by this material the letter "U," for "used," is placed after the number of each of these monkeys in the tables. The weights of monkeys are omitted from the tables, as moderate fluctuations seemed to play no noticeable part in determining the results of the tests. Specimens of monkey blood sent us from Lagos, Nigeria, are included in the tables and identified by specimen numbers preceded by the letter "S" instead of the numbers of the monkeys from which the virus was obtained.

The amounts of blood or liver are stated in the tables in terms of the fresh material. For example, 0.02 gm. of dried blood would be recorded as 0.1 cc., because blood loses about 81 per cent of its weight in drying. 2 cc. of glycerinated blood containing 1 cc. of actual blood would be recorded as 1.0 cc.

Incubation periods are shown in the tables as the interval between inoculation and the first day of fever; for example, if an animal was inoculated in the afternoon and its first fever occurred in the afternoon of the second day after inoculation, the interval would be recorded as 2.0 days, but if the first fever was in the morning of the third day, the period would be 2.5 days. The interval between inoculation and the time of death or recovery is similarly recorded. In the case of recovery the interval is measured to the last recorded temperature of 40°C. or over, and is followed by the letter "R." All deaths recorded were from yellow fever.

In order to limit as far as possible the number of monkeys needed for our investigations, most of the tests of preserved virus for infectivity were made as required by other experiments. This will explain the apparent lack of plan in some of the groups of observations presented.

TABLE I

*Preservation of Yellow Fever Virus in Citrated, Clotted, or Glycerinated Blood*

| Method of preservation | Source, monkey and strain | Amount, blood, fresh | Age of specimen | Monkey inoculated | Interval in days to | | Observation period | Result of test inoculation |
|---|---|---|---|---|---|---|---|---|
| | | | | | Fever | Death or recovery | | |
| | | *cc.* | *days* | *No.* | | | *days* | |
| Citration | 95 A | 0.6 | 0 | 99 | 1.5 | 5.0 | | |
| | 95 A | 0.08 | 0 | 100 | 2.0 | 4.5 | | |
| | 95 A | 0.005 | 0 | 101 | 10.0 | 11.0 | | |
| | 118 F | 0.9 | 1 | 129 | 6.0 | 7.0 R | | Immune |
| | 118 F | 0.07 | 1 | 117 | 2.0 | 4.0 | | |
| | 118 F | 0.006 | 1 | 119 | —* | 8.5 | | |
| | 18 F | 0.6 | 22 | 31 U | — | — | 24 | Not tested |
| | 32 F | 3.5 | 22 | 29 U | — | — | 24 | Not tested |
| | 66 F | 2.0 | 30 | 112 | — | — | 30 | Succumbed |
| | S 103 A | 1.0 | 35 | 42 | — | — | 28 | Not tested |
| | S 104 A | 1.0 | 35 | 45 | 3.5 | 5.5 | | |
| | S 105 A | 1.0 | 35 | 43 | — | — | 28 | Not tested |
| Clotting | 66 F | 2.0 | 30 | 111 | — | — | 30 | Succumbed |
| | S 106 A | 1.0 | 35 | 46 | 21.0 | 24.5 | | |
| Glycerination | 66 F | 2.0 | 30 | 105 U | 6.5 | 10.5 | | |
| | 117 F | 1.0 | 60 | 181 | 12.0 | 16.5 | | |
| | 65 A | 1.0 | 100 | 179 | — | — | 29 | Succumbed |

* No fever was observed before the temperature became subnormal, 6.5 days after inoculation.

### *Citrated and Clotted Blood*

The most convenient method of making direct transfers of yellow fever virus from monkey to monkey was to prevent clotting by the addition of sodium citrate to the freshly drawn blood and to inject the mixture intraperitoneally or subcutaneously. On the day of the bleeding, or even the next day, the virus content of the citrated blood seemed to remain high, as illustrated by the first six items in Table I.

With longer storage the results became increasingly less dependable, and in the six tests of material from 22 to 35 days old there was only one successful inoculation (Table I).

In preparing the citrated specimens, from two to five parts of freshly drawn blood were mixed with one part of sterile 2 per cent sodium citrate solution, usually in 0.9 per cent sodium chloride or Locke's solution. The specimens were then stored in the refrigerator at from 1° to 4°C. The three citrated specimens received from Lagos (S 103, S 104, and S 105 in Table I) consisted of the blood of the same monkey plus an equal quantity of 2 per cent citrate solution. The specimen which proved to be infectious after the long journey from Lagos to New York was one of the two (S 103 and S 104) which had been kept in the ship's refrigerator. The other specimen of the same blood (S 105) had been stored at a higher temperature in the cool room.

If infectious blood is drawn and allowed to clot, it will retain its virulence for about the same length of time as if it had been citrated, as suggested in Table I by the one failure after 30 days in storage and the one success after 35 days. The infectious specimen of clotted blood (S 106) had come from Lagos and was from the same bleeding as the citrated specimens S 103, S 104, and S 105. It had been stored in the ship's cool room. It will be noticed that the incubation period in the case of the successful inoculation with clotted blood was 21 days, the longest in our experience.

Although the virus can be kept in clotted blood about as long as in citrated blood, citration is the more convenient method, for it is necessary to grind the clot with sand and physiological salt solution in a mortar before inoculation. If there is a sufficient amount of serum, this can be used without the clot.

In our experience it appears to be the exception for stored citrated or clotted yellow fever blood to retain its virulence as long as a month, and the method is obviously unsuited for prolonged storage. We made no experiments to determine whether the virus would persist longer in citrated blood under vaseline or oil, or in clotted blood kept continually frozen.

### *Glycerinated Blood or Liver*

As glycerine had proved useful in the preservation of a number of viruses, we made preparations of blood and liver containing 50 per cent of glycerine and tested them for the presence of active virus after storage in a refrigerator at 2° to 6°C. The results are shown in Tables I and II.

The blood specimens were prepared by adding freshly drawn blood to an equal volume of glycerine in a test tube and shaking vigorously. The mixture was a thick fluid which usually clotted gradually in storage until it was of a firm, jelly-

like consistency and required grinding with sand to bring it into solution in physiological salt solution. The liver preparations were of two kinds. The second specimen of glycerinated liver listed in Table II consisted of pieces of liver tissue

TABLE II

*Preservation of Yellow Fever Virus in Frozen, Glycerinated, or Dried Liver*

| Method of preservation | Source, monkey and strain | Amount, liver, fresh | Age of specimen | Monkey inoculated | Interval in days to | | Observation period | Result of test inoculation |
|---|---|---|---|---|---|---|---|---|
| | | | | | Fever | Death or recovery | | |
| | | *gm.* | *days* | *No.* | | | *days* | |
| Freezing | 65 A* | 1+ | 12 | 89 | — | — | 48 | Immune |
| | 40 F | 1+ | 15 | 66 | 6.5 | 9.0 | | |
| | 18 F | 1+ | 18 | 41 | 8.5 | 9.5 R | | Not tested |
| | 32 F | 3+ | 20 | 40 | 5.5 | 8.5 | | |
| | 97 A | 1+ | 21 | 132 | 4.5 | 7.0 | | |
| | 66 F | 1.0 | 30 | 124 | 5.0 | 7.5 | | |
| | 55 A* | 1.0 | 30 | 106 | — | — | 30 | Not tested |
| | 66 F | 1.0 | 100 | 191 | — | — | 30 | Not tested |
| Glycerination | 66 F | 1.0 | 30 | 126 | 6.0 | 11.0 | | |
| | 66 F | 1.0 | 30 | 125 | 12.5 | 15.0 R | | Immune |
| | 117 F | 1.0 | 60 | 187 | 9.0 | 12.0 R | | Immune |
| | 66 F | 1+ | 100 | 193 | 1.5 | 5.0 R | | Not tested |
| Drying | 32 F | 1.0 | 9 | 37 | 19.0 | 22.5 | | |
| | 32 F | 0.1 | 9 | 24 U | 5.5 | 8.0 | | |
| | 32 F | 0.01 | 9 | 14 U | — | — | 30 | Not tested |
| | 66 F | 0.1 | 30 | 127 | 10.0 | 14.5 | | |
| | 32 F | 0.1 | 60 | 94 | — | — | 30 | Not tested |
| | 117 F‡ | 1.0 | 63 | 168 U | — | — | 23 | Succumbed |
| | 32 F | 1.0 | 100 | 146 | 7.0 | 8.0† | | |
| | 66 F | 1.0 | 100 | 192 | 4.0 | 5.0 | | |
| | 32 F | 1.0 | 150 | 190 | 6.5 | 8.0 | | |

* No control inoculation was made with the original liver tissue of this monkey to prove it infectious.

† Chloroformed early in the disease to secure material for pathological study.

‡ Tubed with calcium chloride.

dropped into 50 per cent glycerine in Locke's solution. The other specimens were prepared by grinding liver in an equal volume of Locke's solution, using sand, and then adding to the resulting suspension an equal volume of glycerine.

The results with glycerinated blood were distinctly better than with citrated or clotted blood, as will be seen in the tables. The samples of glycerinated blood

which had been kept 30 and 60 days were shown to contain active virus, but the incubation period for the 60-day specimen was prolonged. The 100-day specimen failed to infect and produced no immunity.

With the liver specimens we obtained infection in every instance, but recovery followed infection from one of the two 30-day specimens and from the 60-day and 100-day specimens.

The virus of yellow fever apparently can be preserved in 50 per cent glycerine for a longer time than in citrated or clotted blood. Infection has even been obtained with glycerinated material after storage for 100 days, but there is a distinct falling off in virulence with storage after 30 days. Stronger concentrations of glycerine have not been tried.

*Frozen Liver*

The method of preserving the virus in frozen liver proved to be reliable for periods of at least one month (Table II); but the single specimen tested after 100 days failed to infect. Pieces of liver in test tubes were placed in the freezing compartment of an ordinary household electric refrigerator. As the temperature in the compartment was $-12°C$. the liver tissue was frozen hard.

On one occasion the refrigerating machinery did not function and the temperature in the compartment rose sufficiently to permit the liver tissue to thaw. As a result the specimens then in storage, the first four in Table II and also the seventh, were under refrigeration for two days without being frozen. The freezing method is simple and valuable when machinery is at hand for maintaining a constant temperature low enough to keep the tissues frozen hard.

*Dried Blood or Liver*

It was not until we tried drying blood or liver under vacuum in the frozen state and storing it in sealed tubes that we were able to preserve the virus of yellow fever for long periods.

The method was suggested to us by Dr. T. M. Rivers, who had found it satisfactory in the preservation of several viruses including vaccine virus and Virus III. What was essentially the same method had been used by Harris and Shackell (4) in 1911 in preserving rabies virus; and Rous (5) reported that in the same year the active virus of a transmissible sarcoma of fowls was stored successfully in dried and powdered tissue by Murphy. More recently the method of drying while in the frozen state was applied to the preservation of bacterial cultures by Swift (6).

Our usual procedure in preserving the yellow fever virus in dried blood commences with the drawing of 15 to 20 cc. of blood from the heart of a monkey under ether anesthesia on the first day of fever. The needle of the syringe is then thrust through several layers of gauze which cover a sterile, cylindrical glass evaporating dish 12 cm. in diameter and 6.5 cm. deep, and the blood is ejected. The thin layer of fluid blood on the bottom of the dish is then quickly frozen by setting the dish in a shallow pan containing alcohol and pieces of solid carbon dioxide.

At least an hour before the bleeding, a desiccator of the Hempel improved type with an internal diameter of about 15 cm. is packed in an ice-salt mixture in a rectangular metal pan. In the groove in the upper part of the desiccator is about 130 cc. of fresh concentrated sulphuric acid to absorb the moisture given off by the blood. In the bottom is enough glycerine to cover a porcelain platform and make a good contact with the evaporating dish.

When the blood in the evaporating dish is thoroughly frozen, the dish is lifted from the alcohol, the liquid is quickly wiped from the bottom, and the dish is placed in the desiccator. The cover of the desiccator is then given at least one complete turn to make sure that a good contact is made. An extremely small quantity of a suitable thick lubricant has been applied to the contact surfaces in advance. The desiccator is then evacuated with an electric air pump until the pressure has fallen to 1 or 2 mm. of mercury. The desiccator in its pan of ice and salt is put into a refrigerator to prevent rapid melting of the ice. From 16 to 20 hours later the vacuum is released and the desiccator is opened. The evaporating dish is taken out, wiped free of glycerine, and placed on a sheet of paper under a small glass-topped frame. The object of the frame is to prevent the distribution, by air currents, of infectious dust which might be inhaled by the operator. Rubber gloves are worn while the dried material is being tubed.

About ten plugged sterile test tubes measuring 12 by 200 mm. are numbered and weighed in advance. They are taken one by one, warmed slightly in the flame to prevent condensation of moisture, partially filled with the flakes of dried blood, flamed at the mouth, replugged, and again weighed. The gain in weight in grams represents the weight of the dried blood, which is approximately one-fifth of the weight of the fresh blood or of its volume in cubic centimeters. In the evaporating dish the dried blood is in the form of a thin, red, brittle wafer, with fissures running through it. It lies loose from the glass and can easily be broken into bits and transferred to the test tubes with forceps and narrow spatula. It is light and porous and can be readily crushed in a mortar to a smooth powder before it is tubed, but this probably increases the risk of accidental infection and ordinarily is of no advantage. After the weighing, the tops of the plugs are cut off with scissors and the remainder pushed down to the surface of the dried blood with a rod. To make certain of complete dryness, granular calcium chloride is poured in above the plugs before the tubes are sealed in the flame of a blast lamp. The tubes are stored in the refrigerator. The use of calcium chloride, although

perhaps advisable, is not necessary, since the oldest specimens tested were put up without it. The method of putting in the calcium chloride has been described by J. H. Brown (7).

When the dried blood is required for inoculation, the tube is opened by scratching with a file at the middle of the cotton plug and touching with a hot piece of glass or metal. The plug is carefully removed and a small amount of 2 per cent sodium citrate in physiological salt solution is put in to prevent scattering of dust. The contents are then emptied into a mortar, the adherent particles are scraped out of the tube with a platinum wire, and the material is ground up with additional fluid. Without the sodium citrate in the physiological salt solution the dissolved blood will clot in the mortar and make injection difficult.

Small quantities of blood, 1.0 cc. or 0.5 cc., are easily dried in a test tube or ampoule. The dry blood forms a small button which lies loose in the bottom of the container. The blood may be put up in ampoules with safety and with great simplicity of technique, but this advantage over the use of the evaporating dish and test tube is more than offset by the greater difficulty of getting the material out.

Where solid carbon dioxide ("dry ice") is not available the blood may be frozen in small amounts (1.0 or 0.5 cc.) in a number of test tubes in an ice-salt mixture before desiccation.

To preserve the virus in liver tissue, from 10 to 20 gm. of the fresh tissue is ground with sterile sand in a mortar without added fluid. The resulting paste is scraped into the glass evaporating dish and the sterile gauze cover is replaced and tied with string. Gentle tapping of the dish on a table top will complete the even distribution of the material over the bottom of the dish. The material is then frozen, dried, and sealed in tubes by the same technique as the one used for blood. As the total weight of the original material is known, the amount of liver tissue in each tube, in terms of fresh liver, is determined by dividing the original weight of the liver in proportion to the final weights of liver and sand in the several tubes. Inoculations are made with the material as in the case of blood, but physiological salt solution is used instead of citrate solution. The dried liver is soft, porous, and friable, and has a light greyish yellow color due to the presence of much fat.

## Freezing is not absolutely necessary for the successful preservation of blood.

Specimen 157 A (Table III) was prepared in Lagos by the West African Yellow Fever Commission of the Rockefeller Foundation and was brought to us through the courtesy of Dr. Oskar Klotz of the Commission. Hudson and Klotz had demonstrated that infected monkey blood, desiccated in a vacuum in the presence of sulphuric acid or calcium chloride, at room temperature or in the icebox, remained infective as long as 38 days (8). In preparing Specimen 157 A the mixed blood of two monkeys was dried in a vacuum over calcium chloride and sealed

TABLE III

*Preservation of Yellow Fever Virus in Dried Blood*

| Method of preservation | Source, monkey and strain | Amount, blood, fresh | Age of specimen | Monkey inoculated | Interval in days to | | Observation period | Result of test inoculation |
|---|---|---|---|---|---|---|---|---|
| | | | | | Fever | Death or recovery | | |
| | | *cc.* | *days* | *No.* | | | *days* | |
| Drying | 180 A*‡ | 2.5 | 12 | 202 | — | — | 32 | Not tested |
| | 180 A*‡ | 0.5 | 18 | 205 | — | — | 32 | Not tested |
| | 65 A | 0.1 | 13 | 95 | 3.5 | 7.5 | | |
| | S 86 A¶ | 1.0 | 28 | 28 | 7.0 | 9.5 | | |
| | S 87 A¶ | 1.0 | 28 | 29 | — | — | 30 | Not tested |
| | 66 F | 0.1 | 30 | 118 | 4.0 | 6.0 | | |
| | 55 A | 0.1 | 31 | 107 | 6.0 | 10.0 | | |
| | S 157 A¶ | 6+ | 32 | 103 | 3.5 | 7.5 | | |
| | 117 F‡ | 0.3 | 42 | 144 U | —† | 4.0 | | |
| | 117 F‡ | 0.3 | 42 | 143 U | 2.5 | 4.5 | | |
| | 117 F‡ | 0.3 | 42 | 145 U | 4.0 | 7.5 | | |
| | 66 F | 0.5 | 60 | 147 U | 3.5 | 7.5 | | |
| | 117 F‡ | 0.5 | 60 | 157 U | 1.5 | 3.0 | | |
| | 65 A | 0.1 | 61 | 150 | 3.0 | 4.5 | | |
| | 55 A | 0.1 | 78 | 152 | 3.0 | 5.0 | | |
| | 117 F‡ | 1.0 | 86 | 209 | 2.0 | 2.0‖ | | |
| | 117 F‡ | 1.0 | 94 | 188 U | 1.5 | 3.5 | | |
| | 65 A | 0.1 | 100 | 180 | 3.5 | 10.0 | | |
| | 66 F | 0.1 | 100 | 188 | — | — | 30 | Succumbed |
| | 55 A | 0.1 | 101 | 176 | 9.0 | 11.0 | | |
| | 55 A | 0.01 | 101 | 177 | — | — | 37 | Succumbed |
| | S 157 A§¶ | 0.1 | 102 | 173 | 3.5 | 5.0 | | |
| | 55 A | 1.0 | 109 | 151 U | 3.0 | 5.5 | | |
| | 65 A | 1.0 | 127 | 210 | — | — | 30 | Not tested |
| | 55 A | 1.0 | 154 | 235 | 4.0 | 6.5 | | |
| | S 157 A§¶ | 1.0 | 155 | 231 | 16.5 | 19.0 | | |

* This specimen, when 6 days old, produced fever in a monkey in 2 days and death from yellow fever in 3.5 days.

† No fever observed.

‡ Tubed with calcium chloride. The other tubes were without it.

§ Re-tubed with calcium chloride when 43 days old.

‖ Killed to obtain material for study of early lesions.

¶ Dried without freezing.

TABLE IV

*Groups of Specimens of the Same Material Preserved in Different Ways and for Varying Lengths of Time*

| Source, monkey and strain | Blood | | | | Liver | | | |
|---|---|---|---|---|---|---|---|---|
| | Method of preservation | Amount, fresh | Age of specimen | Result. Yellow fever? | Method of preservation | Amount, fresh | Age of specimen | Result. Yellow fever? |
| | | *cc.* | *days* | | | *gm.* | *days* | |
| 32 F | | | | | Freezing | 3+ | 20 | + |
| | | | | | Drying | 1.0 | 9 | + |
| | | | | | | 0.1 | 9 | + |
| | | | | | | 0.01 | 9 | − |
| | | | | | | 0.1 | 60 | − |
| | | | | | | 1.0 | 100 | + |
| | | | | | | 1.0 | 150 | + |
| 55 A | Drying | 0.1 | 31 | + | | | | |
| | | 0.1 | 78 | + | | | | |
| | | 0.1 | 101 | + | | | | |
| | | 0.01 | 101 | − | | | | |
| | | 1.0 | 109 | + | | | | |
| | | 1.0 | 154 | + | | | | |
| 65 A | Glycerine | 1.0 | 100 | − | | | | |
| | Drying | 0.1 | 13 | + | | | | |
| | | 0.1 | 61 | + | | | | |
| | | 0.1 | 100 | + | | | | |
| | | 1.0 | 127 | − | | | | |
| 66 F | Citration | 2.0 | 30 | − | Freezing | 1.0 | 30 | + |
| | Clotting | 2.0 | 30 | − | | 1.0 | 100 | − |
| | Glycerine | 2.0 | 30 | + | Glycerine | 1.0 | 30 | + |
| | Drying | 0.1 | 30 | + | | 1.0 | 30 | + R |
| | | 0.5 | 60 | + | | 1+ | 100 | + R |
| | | 0.1 | 100 | − | Drying | 0.1 | 30 | + |
| | | | | | | 1.0 | 100 | + |
| 117 F | Glycerine | 1.0 | 60 | + | Glycerine | 1.0 | 60 | + R |
| | Drying | 0.3 | 42 | + | Drying | 1.0 | 63 | − |
| | | 0.5 | 60 | + | | | | |
| | | 1.0 | 86 | + | | | | |
| | | 1.0 | 94 | + | | | | |

in a glass tube. It was transported from Africa to America at room temperature. A part was used in a successful inoculation when the material was 32 days old. The remainder was retubed with calcium chloride and was found to be highly virulent when 102 days old (Table III). It infected also after 155 days, but the incubation period was prolonged. The dried blood of this specimen consisted of small black glassy scales in striking contrast to the soft porous red pieces of the blood which was frozen before being dried. Specimens 86 and 87, from Lagos, were also dried without preliminary freezing. One of the two was found to be infectious when 28 days old (Table III), but the other was inert.

The specimens of blood and liver which had been frozen and dried seldom failed to produce infection. Two of the three specimens of blood which were tested when 100 days old were found to be virulent when injected in amounts as small as the equivalent of 0.1 cc. of fresh blood. One of these two failed to infect at 127 days. The other was tested again when 154 days old, in a dosage equivalent to 1.0 cc. and produced yellow fever after an incubation period of only four days. Tests after longer periods have not yet been made.

The dried liver gave similar results, but the observations were fewer (Table II). Our oldest specimen (from Monkey 32) failed to infect when 60 days old when a dose equivalent to 0.1 gm. of fresh liver was injected, but infected after 100 days and after 150 days when the equivalent of 1.0 gm. was injected. Another specimen 100 days old was tested and found to be infectious.

The process of freezing and drying will preserve bacteria as well as the virus of yellow fever, and one would expect to get bacterial infections occasionally from the use of dried liver, since pathogenic organisms frequently invade the liver late in the disease. Such infections would occur very seldom when dried blood is used. As the result of inoculation with a specimen of fresh yellow fever liver we lost two monkeys from a streptococcus peritonitis, and inoculation with another specimen, which had been sent us under refrigeration, caused three animals to develop tuberculous peritonitis.

### *Comparison of Methods*

A close comparison of the value of the methods can only be made by submitting the same original material to the several processes. One specimen of fresh blood or liver may contain many times as much virus as another. We have, therefore, brought together in groups in Table IV the specimens derived from each of several lots of blood or liver. Each of these specimens appears also in one of the previous tables, where additional data are given.

## CONCLUSIONS

1. The virus of yellow fever may be preserved for at least 154 days in the blood or liver tissue of infected monkeys if the material is dried

in a vacuum while in the frozen state and kept in the refrigator in sealed glass containers. A gradual diminution of virulence is noticeable in the older specimens.

2. If infectious blood is dried in a vacuum at room temperature, instead of in the frozen state, and is stored in sealed containers in the refrigerator, the virus may survive as long as 155 days.

3. The virus may be preserved for at least 30 days in liver kept continuously frozen.

4. Storage of blood or liver in 50 per cent glycerine in the refrigerator will usually keep the virus alive for 60 days and may do so for 100 days, but with the injection of the older material there is a marked tendency toward lengthening of the incubation period and increase in the number of recoveries.

5. Yellow fever virus in citrated or clotted blood, when kept in the refrigerator, dies out rapidly.

6. In our experience the most satisfactory method of preserving strains of yellow fever virus in the laboratory consists of freezing and drying blood taken from a monkey on the first day of an attack of experimental yellow fever and storing the dry material in sealed glass tubes in a cold place.

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